PATENT
Docket No.: **176/61411 (2-11141-03010)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Wu et al.	)	Examiner:
		)	Michail A. Belyavskyi
Serial No. :	10/679,184	)	
		)	Art Unit:
Cnfrm. No. :	2775	)	1644
		)	
Filed :	October 3, 2003	)	
		)	
For :	THREE-DIMENSIONAL PERIPHERAL LYMPHOID ORGAN CELL CULTURES	)	
		)	

REQUEST FOR RECONSIDERATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the August 1, 2006, office action, reconsideration of the outstanding office action is respectfully requested.

Claims 31–120 have been withdrawn. Claims 1–120 are pending.

Peripheral lymphoid organs represent the principal sites of adaptive immune responses and their complex anatomy reflects the presence of well-defined subregions enriched for specific immune system cell subsets. As a model peripheral lymphoid organ, the ultrastructure of the spleen is reviewed in the present application.

In the mammalian spleen, leukocytes localize principally within the so-called white pulp, while the red pulp is predominantly involved in the trafficking and processing of erythrocytes. The white pulp is organized around the afferent vases from which leukocytes enter the spleen, and can be subdivided into 3 main regions, as shown in Figure 1 of the present application. The first area is formed by the follicles, which are highly enriched for mature B cells expressing high surface IgD levels and low IgM (follicular B, FB, or B2 cells), as well as follicular dendritic cells ("FDC"), also involved in antigen capture and presentation. The marginal zone ("MZ") surrounding the follicle is enriched in a population

of B lymphocytes characterized by high levels of expression of IgM and of the CD21 marker; MZ B cells are thought to be involved in primary responses and particularly in responses to T cell helper-independent antigens. Adjacent to the B cell follicle is a T cell-rich zone, the periarterioral lymphoid sheets ("PALS"), which also contains a population of specialized antigen-presenting cells, the dendritic cells ("DC"). During a humoral immune response, specialized structures called germinal centers ("GC") appear in the follicular area. They are the principal sites for several crucial activities of activated B cells: clonal expansion, affinity maturation (the increase in antibody affinity to antigen following hypermutation of antibody genes), and generation of secondary Ig isotypes (class switching). Also, probably in the GC, the crucial terminal B cell differentiation decision is made between the path to long-lived memory B cells and that to antibody-secreting plasma cells. The basic organization of the spleen in segregated B and T cell areas is replicated in lymph nodes, with the exception of the absence of marginal zones.

The highly structured anatomy of peripheral lymphoid organs reflects complex homing, cell-cell interaction, and adhesion processes that are essential for immune system function. Structural disruption of peripheral lymphoid organs, as in the case of mutants for certain TNF-family factors and their receptors, results in an inability to mount fully functional immune responses.

During *de novo* lymphoid organogenesis, hematopoietic fetal liver-derived non-T/non-B precursors with $CD45^+$, $CD4^+$, $CD3^-$ phenotype represent one of the earliest colonizing cells of lymph nodes. These cells are believed to be the main cellular source of lymphotoxin ("LT") $\alpha 1\beta 2$, a cytokine critical for the formation of organized peripheral lymphoid tissues. Interestingly, similar $CD4^+$, $CD3^-$ cells also play an important role in T-B cell cooperation during primary and memory phases of the immune response in the adult organism. Signaling by $LT\alpha 1\beta 2$ through the $LT\beta$ receptor expressed by stromal cells leads to up-regulation of adhesion molecules and production of chemokines, chiefly CXCL13. These, in turn, induce clustering of stromal and hematopoietic cells, initiating the organogenesis process. CXCL13 plays an essential role in these events, as no follicles are formed in lymphoid organs of CXCL13 mutant mice. These early events are followed by further colonization of lymph node structures with diverse hematopoietic subsets (B and T cells, dendritic cells, and macrophages), which also get intimately involved in the organogenesis

process. LTα1β2 exerts further control on the organization and maintenance of the lymphoid organ structure.

Importantly, many of the microenvironmental signals required for lymphoid organogenesis also appear to be necessary and sufficient during *de novo* formation of tertiary lymphoid structures (lymphoid neogenesis), which occurs in some inflammatory processes of infectious or autoimmune origin. Indeed, ectopic expression of transgenic lymphotoxin or BLC chemokine is sufficient to induce lymphoid neogenesis.

B cell activation and terminal differentiation during a humoral immune response represent the integrated result of the temporal, spatial, and kinetic modulation of signals from a number of surface receptors involved in antigen recognition (the B cell receptor), cell-cell interactions (such as CD40, OX40 ligand, adhesion receptors), and detection of soluble lymphokines.

The interpretation of all these signals by the B cell is largely dependent on the microenvironmental context in which they are delivered, as highlighted by the strict localization of the successive steps of the B cell activation process. Thus, during a classical T cell-dependent response, follicular B cells usually encounter antigen within the PALS, in the course of their migration from afferent vases to the B cell follicles. Here, interactions can form between antigen-specific B cells, T cells, and DCs, resulting in a first surge of lymphocyte activation, initial expansion of antigen-specific clones, and the initiation of a primary immune response, which provides a rapid burst in antibody production via the generation of resident, short-lived plasma cells. Lymphocyte clones generated in this first phase leave the PALS and proceed to the follicles, where they initiate the formation of GCs. Within GCs, highly proliferating B cells (centroblasts) expand the pool of antigen-specific cells and undergo somatic hypermutation of their Ig genes, which changes the affinity of the antibody for its antigen. B cells emerging from this phase undergo selection for their affinity to antigen, present in immune complexes on the surface of FDCs. High-affinity clones receive surface Ig-mediated and survival signals, which allow them to further differentiate, after interaction with TH cells, into plasmablasts and memory B cells. These exit the GC and leave the lymphoid organ microenvironment as circulating memory B cells, or as long-lived plasma cells, which usually home back to the bone marrow for long-term antibody production.

Responses to many bacterial, polysaccharide, and highly repetitive antigens do not require T cell help and do not lead to germinal center formation. Even in this case, however, the specific spatial and temporal organization and interaction of individual cell subsets (most notably, MZB and B-1 cells) within restricted anatomical compartments (such as the MZ) is necessary for the development of an immune response.

The delivery of an activation signal is required for survival of B cells *in vitro*. In the absence of such signals, >90% of follicular B cells undergo apoptosis within 72 hours of culture, a process that can be inhibited by transgenic expression of antiapoptotic genes, such as bcl-2. Interestingly, rapid apoptosis of murine mature B cells following deletion of their Ig coding sequences in inducible Cre-Lox transgenic mice suggests that low-level, "tonic" signals from the BCR are also likely required for survival of peripheral B lymphocytes *in vivo*. B1 cells appear to have somewhat different *in vitro* kinetics, surviving in greater numbers during the first few days of culture (about 50% at day 7), then undergoing a crisis from which isolated long-lived, clonal populations can arise after 6–8 weeks. Such clones, although non-tumoral, appear to be able to propagate indefinitely *in vitro* and display some molecular hallmarks of transformed cells, including the amplification of c-myc. The long-term maintenance of resting B lymphocytes in the absence of over-expression of proto-oncogenes is therefore unfeasible using current technology.

Splenocytes from mice that have been previously antigen-primed (*i.e.*, immunized) *in vivo*, but not naïve splenocytes, are able to mount relatively weak but detectable antigen-specific antibody responses *in vitro*. As discussed above, the absence of primary responses in these conditions may be at least in part due to the short life span of resting B lymphocytes *in vitro*. Thus, the more rapid entry into cell cycle of antigen-experienced vs. naïve lymphocytes upon antigen stimulation would allow the former, but not the latter, to become activated before apoptosis occurs. Furthermore, human activated, germinal center B cells can be made to differentiate into memory cells or plasma cells *in vitro* in the appropriate culture conditions. Altogether, these findings suggest that primary, *ex vivo* lymphocytes are competent to respond to specific signals and differentiate into effector and memory cells *in vitro*, but that the lymphoid organ microenvironment provides specific signals for primary immune response initiation, cell survival, and differentiation.

Blood cell formation in normal adults takes place in the extravascular space between bone marrow sinuses. Besides the hematopoietic cells, the marrow also contains

stromal cells including the endothelial cells, reticular cells, and macrophages. The stromal cells, their cell processes, and the extracellular matrices they secrete form a three-dimensional scaffolding upon which the hematopoietic cells lodge. The stromal cells, through their intimate physical contact with the hematopoietic cells, the extracellular matrices, and the growth factors they secrete, create the intricate Hematopoietic Inductive Microenvironment, which regulates the proliferation and differentiation of the hematopoietic cells. It has been hypothesized that "niches," formed by the stromal cells, direct the hematopoietic cells towards self-renewal or differentiation into specific lineages.

Most studies of *in vitro* hematopoiesis have used the murine long-term bone marrow culture ("LTBMC") system, first developed by Dexter and co-workers employing tissue culture flasks or bottles. In the Dexter culture, the stromal cells spread and attach to the surface of the culture flask, forming a flat adherent layer. The stromal cells become extremely flattened and are therefore called "blanket cells." The hematopoietic cells loosely bind to the stromal layer (the adherent compartment) where they proliferate and differentiate. The mature blood cells and some of the progenitor cells are released into the culture medium (the non-adherent compartment). Active growth of hematopoietic cells results in hematopoietic foci with clusters of cells, described as "the cobblestone areas" due to their distinct appearance under a phase-contrast inverted microscope. Under these conditions, normal B-lymphoid cells in 2D bone marrow cultures become progressively skewed toward precursor B-cell populations that do not retain a normal immunophenotype, and mature B-lymphocytes, such as those harvested from spleen or lymph node, do not survive beyond several days *ex vivo* in the absence of mitogenic stimulation.

Despite its ability to support hematopoietic differentiation, several limitations are obvious in the Dexter culture system. Most notably, the mature cells produced in the "classic" cultures are mainly neutrophils and monocytes/macrophages (especially in the murine system), and specific alterations have to be introduced to drive differentiation of other cell lineages. In addition, hematopoiesis in the flask culture is accompanied by extensive lipogenesis of the stromal cells and formation of fat cells, which are rarely found in normal human marrow with active hematopoiesis and are virtually absent in the marrow of mouse femur. In addition, the optimal temperature for cell output and duration of Dexter culture is 33°C, a temperature not optimal for growth of all cells.

Peripheral lymphoid organs represent critical sites for the development of adaptive immune responses. They are characterized by a complex histological organization in which specific localization of cell subsets, and their physical interaction and migration during the course of the immune response, are tightly controlled by microenvironmental signals such as cytokines, chemokines, adhesion molecules, and homing receptors. The essential role these factors play in immune system function has hampered the faithful replication of normal immune mechanisms *in vitro*. Indeed, even the long-term culture of peripheral lymphocytes in the absence of activating stimuli or cell transformation has not been achieved so far.

The present invention is directed to overcoming these and other deficiencies in the art.

The rejection of claims 22 and 23 under 35 U.S.C. § 112 (2nd para.) for indefiniteness is respectfully traversed.

The United States Patent and Trademark Office ("PTO") asserts at page 3 of the office action that claims 22 and 23 are unclear for three reasons. The first is that it is unclear whether cells are isolated and reseeded into a "new" culture medium or whether cells with "old" culture medium are reseeded. The second concerns whether the reseeding step requires the three-dimensional matrix. The third is that essential steps of contacting, detecting, and correlating are not recited. Applicants respectfully disagree with each of these assertions.

Claims 22 and 23 do not recite "new" and "old" culture mediums—"the culture medium" recited in claims 22 and 23 clearly refers back to the "culture medium" of claim 1 (*i.e.*, as recited in the phrase "covered or surrounded with culture medium"). Likewise, the reseeding step applies to the "culture medium" of claim 1, which covers or surrounds the three-dimensional matrix. Thus, the first and second bases of rejection are improper.

As for the third basis of rejection, the present claims are not directed to an assay—not only are contacting, detecting, and correlating steps not required, they are inapposite to the present claims. Thus, the third basis of rejection is improper.

For all these reasons, the rejection of claims 22 and 23 for indefiniteness should be withdrawn.

The rejection of claims 1–30 under 35 U.S.C. § 102(a) for anticipation by WO 01/036589 to Wu et al. ("Wu") is respectfully traversed.

Wu teaches a cell culture system that includes a three-dimensional support for the culture of hematopoietic stem cells and stromal cells, and media that supports the growth or differentiation of the stem cells into immune system cells. Wu specifically describes culturing the mononuclear cell layer of human bone marrow in a three-dimensional bioreactor, and reports that the culture resembled the function of bone marrow *in vivo*. *See* Wu at 32–40.

The PTO's position is that Wu teaches a method of culturing peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells. Applicants respectfully disagree.

The position of the PTO appears to rest on the assumption that the bone marrow-derived hematopoietic stem cells of Wu are peripheral lymphoid organ cells. This is not the case.

In support of applicants' position that the PTO's assumption is incorrect, accompanying this response is the Declaration of Andrea Bottaro Under 37 C.F.R. § 1.132 ("Bottaro Decl."). Bone marrow is the primary hematopoietic organ in mammals. Bottaro Decl. ¶ 6. Hematopoietic cells in bone marrow are phenotypically and functionally distinct from hematopoietic cells in the periphery (*i.e.*, in blood, lymph, peripheral lymphoid organs, and other tissues). *Id.* These differences are especially crucial in the case of hematopoietic cells involved in immune responses, because these cells *must* complete their maturation in the periphery to become fully functional, as highlighted below. *Id.*

Briefly, the bone marrow is the site where hematopoietic cells (lymphocytes; myelocytes, *i.e.*, granulocytes, monocyte/macrophages, platelets and certain kinds of dendritic cells; and red blood cells) differentiate from common precursor stem cells. Bottaro Decl. ¶ 7. This initial stage of differentiation leads to generation of immature cells that leave the bone marrow and enter the periphery. *Id.* In the case of lymphocytes (B and T cells), the principal effectors of adaptive immune responses in mammals, the maturation is only completed after exiting the bone marrow. *Id.* T cell precursors from the bone marrow need to undergo a complex maturation process in the thymus, where they are selected for their ability to discriminate histocompatibility antigens on the surface of other cells. *Id.* B cells must also complete their maturation in the periphery, also becoming competent in their ability

to distinguish foreign substances from "self." *Id.* These maturation steps involve significant changes in the phenotype of cells (expression of specific molecules on the cell surface) as well as in their functional properties (ability to respond to stimuli, "homing" to specific tissues and sites, etc.). *Id.* For instance, B lymphocytes newly generated from the bone marrow respond to stimulation of their surface antibody molecules by either dying (apoptosis) or by becoming inactivated, but the same stimulus delivered to a mature B cell triggers cell activation and involvement in an immune response. *Id.* Disruption of these peripheral maturation processes can lead to immunodeficiency or, at the other end of the spectrum, autoimmunity. *Id.* Therefore, there are critical differences between the intrinsic features of hematopoietic cells, and especially immune system cells, residing in the bone marrow, and those residing in peripheral lymphoid organs. *Id.* In short, hematopoietic stem cells derived from bone marrow are not peripheral lymphoid organ cells. *Id.*

Moreover, a skilled scientist familiar with Wu would not have expected to be able to culture peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells. Bottaro Decl. ¶ 8. That is because salient differences exist in the ultra-structural and histological organization of peripheral lymphoid organs (*e.g.*, spleen, lymph nodes) and bone marrow, which reflect their different functions. *Id.* The role of the bone marrow stroma is to support and sustain the maturation process of the hematopoietic precursors along various cell lineages, including lymphocytes. *Id.* Stromal cells in the peripheral lymphoid organs have the role of organizing the histology of the organ in specific zones as required for functional immune responses to develop. *Id.* In both the spleen and lymph nodes, specialized areas are identifiable where T cells and various subsets of B cells specifically reside (periartherioral lymphoid sheaths, B cell follicles, and marginal zones). *Id.* These areas have no counterpart in the bone marrow, and correspond to the distribution of specific stromal cells (such as follicular dendritic cells) that develop *in situ* by effect of the mature lymphocytes themselves. *Id.* This structural organization is key in generating and sustaining immune responses *in vivo*, in that the migration of antigen-specific lymphocytes in the appropriate areas where antigen may be presented to them by accessory cells, and their proliferation and differentiation, are tightly regulated processes whose disruption can lead to defective immune responses. *Id.* Finally, immune responses culminate in the generation, within the structure of peripheral lymphoid organs, of unique histological sites called germinal centers, in which antigen-specific B cells,

T cells, and follicular dendritic cells cooperate in the generation of both effector cells that mediate immune responses, as well as memory cells responsible for long-term immunity to pathogens (*e.g.*, following vaccine inoculation). *Id.* Therefore, crucial differences exist between peripheral lymphoid organs and bone marrow in their histological, functional, and cellular properties. *Id.*

For the reasons highlighted above, the behavior of hematopoietic lineage cells from peripheral lymphoid organs within a bioreactor microenvironment was neither directly expected nor predictable based on the methods of Wu with respect to bone marrow hematopoietic cells. Bottaro Decl. ¶ 9. Peripheral lymphoid organ cells are exquisitely sensitive to specific microenvironmental signals delivered by both cell-cell interactions with cognate cells, as well as by soluble factors permeating the various areas within the peripheral lymphoid organ, and disruption of these signals in culture conditions normally leads to rapid death or acquisition of unusual and potentially dysfunctional phenotypes. *Id.* Therefore, the success in culturing bone marrow hematopoietic cells in a three-dimensional bioreactor as described in Wu would not have lead a scientist to conclude that one could culture peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells. *Id.*

The finding of the present application that bioreactor cultures are able to sustain for several weeks the presence of various subsets of peripheral lymphoid organ cells, which closely replicate, both phenotypically and functionally, those found in normal, intact peripheral lymphoid organs, is not disclosed in Wu. Bottaro Decl. ¶ 10.

For these reasons, the rejection of claims 1–30 for anticipation by Wu is improper and should be withdrawn.

The rejection of claims 1–7 and 13–23 under 35 U.S.C. § 102(b) for anticipation by WO 99/15629 to Pykett et al. ("Pykett") or U.S. Patent No. 5,160,490 to Naughton et al. ("Naughton") is respectfully traversed.

Pykett has been cited for teaching a method of culturing, on a three-dimensional porous biomaterial, hematopoietic progenitor cells in the absence of exogenously added hematopoietic growth factors. Pykett specifically describes carrying out its method using hematopoietic progenitor cells derived from human bone marrow. *See, e.g.*, Pykett at 26–27. Naughton teaches a method for culturing cells and tissues *in vitro* for prolonged periods of time. This method involves culturing cells derived from a desired tissue

on a pre-established stromal support matrix. Naughton specifically describes carrying out its method with bone marrow cells, skin cells, liver cells, mucosal epithelial cells, pancreatic cells, brain cells, and adenocarcinoma cells. *See, e.g.*, Naugton at §§ 11–17.

The PTO has taken the position that Pykett and Naughton teach culturing peripheral lymphoid organ cells under conditions effective to generate and maintain mature and functional peripheral lymphoid organ cells. Applicants respectfully disagree for substantially the reasons noted above with respect to Wu. In particular, as noted above, the bone marrow-derived hematopoietic progenitor cells of Pykett are not peripheral lymphoid organ cells, nor are the cells disclosed in Naughton. Therefore, the rejection of claims 1–7 and 13–23 for anticipation by Pykett and Naughton is improper and should be withdrawn.

The rejection of claims 1, 6, 8–12, and 24–30 under 35 U.S.C. § 103(a) for obviousness over Pykett or Naughton in view of U.S. Patent No. 6,821,778 to Engleman et al. ("Engleman") and U.S. Patent No. 6,274,378 to Steinman et al. ("Steinman") is respectfully traversed. (The outstanding office action refers to "U.S. Patent '378" at page 5, paragraph 7, through page 6, paragraph 2. It is assumed that the PTO intended to reference Steinman as a basis for this obviousness rejection.)

The deficiencies of Pykett and Naughton are recited above.

Engleman relates to a method for preparing activated antigen-specific γδ-TCR^+ human T cells *in vitro* by co-culturing antigen-presenting human dendritic cells with γδ-TCR^+ human T cells so that the T cells are activated to proliferate or to become cytotoxic in response to the antigen.

Steinman relates to a method of preparing large numbers of stable, mature dendritic cells, and teaches that antigen-presenting dendritic cells prepared according to its method are useful for activating T-cells.

The PTO's position is that it would have been obvious in light of Engleman and Steinman to include antigen-presenting dendritic cells in the media of Pykett and Naughton. However, neither of these references overcomes the deficiencies of Pykett and Naughton noted above, and the PTO has failed to demonstrate how these deficiencies are overcome by these secondary references. Therefore, the rejection of claims 1, 6, 8–12, and 24–30 for obviousness over Pykett, Naughton, Engleman, and Steinman is improper and should be withdrawn.

The provisional rejection of claims 1–30 on the ground of nonstatutory obviousness-type double patenting over claims 1–120 of U.S. Patent Publication No. 2005/0191743 to Wu et al. ("the '743 application") and claims 1–106 of U.S. Patent Publication No. 2003/0109042 to Wu et al. ("the '042 application") is respectfully traversed.

The '743 application is the published form of the present application, while the '042 application has been abandoned. Since the PTO has failed to base its provisional double patenting rejection on the claims of a co-pending application, this rejection is improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: 1 February 2007

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